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In vivo footprinting was utilized to examine the estrogen-responsive pS2 gene in MCF-7 human breast cancer cells. Estrogen treatment resulted in an extensive region of protection within and adjacent to the pS2 estrogen response element (ERE). The ERE was also strongly protected after addition of either 4-hydroxytamoxifen and ICI 182,780, but the pattern of protection was distinct with each of these antiestrogens. Protein-DNA interactions in several other regions of the pS2 promoter were also differentially affected by estrogen and antiestrogen treatment. The TATA and CAAT sequences in the proximal promoter were protected in MCF-7 cells in the presence and in the absence of hormone, but were flanked by hypersensitive sites, which were unaffected by hormone treatment. Hypersensitive sites, which were observed in the region of the pS2 ERE in MCF-7 cells, were not observed in estrogen receptor (ER)-negative MDA MB 231 cells and the overall organization of the gene was distinctly different. Taken together, these findings imply that the ER is involved not only in mediating the cellular response to hormone, but may also be involved in organization of the proximal promoter in the absence of hormone.

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Larry N. Petz 8/21/98

PI - Signature Date

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INTRODUCTION

Estrogen is a hormone of critical importance in the development and maintenance of normal reproductive tissues. Estrogen's actions are mediated through an intracellular estrogen receptor (ER), which interacts with estrogen response elements (EREs) in target genes to initiate changes in transcription (1). Much of the work examining the ER-ERE interaction has been carried out using *in vitro* binding studies or transient transfection assays. While these studies have provided us with a great deal of information about the ER-ERE interaction, they utilize linear DNA fragments and supercoiled plasmids which fail to take into account the role of chromatin in regulating gene transcription.

In addition to its role in normal cell maintenance, estrogen has also been implicated in initiation and proliferation of mammary tumors. One estrogen-responsive gene, which is expressed in many breast cancer cells, but not in normal mammary cells is the pS2 gene. The function of the pS2 protein in mammary tumors is not known, but may be involved in regulating cell growth (2). In fact, levels of this protein have been used as a marker of estrogen responsiveness in ER-containing breast cancer cells (2-4) and an indicator of disease progression (5, 6). Exposure of MCF-7 human breast cancer cells to 17β-estradiol (E_2) activates transcription of the pS2 gene and results in increased levels of pS2 mRNA and secreted protein (2, 6-8). The pS2 gene contains all of the elements of a classic hormone-responsive gene: an ERE, a TATA box, and a CAAT box (Fig. 1). Transient transfection assays have been used to demonstrate that the imperfect ERE sequence confers estrogen-responsiveness to this gene (9).

To understand how native estrogen-responsive genes are regulated in living cells, we have utilized *in vivo* footprinting to examine regulatory elements of the pS2 gene as they exist in

nucleosomally phased chromatin residing in intact MCF-7 cells. MCF-7 cells, which were derived from a metastatic breast cancer pleural effusion (10), maintain substantial levels of ER (11) and their responsiveness to estrogen has been well documented (7, 8, 12, 13). Thus, the MCF-7 pS2 gene provides us with an ideal model system to define mechanisms involved in regulation of estrogen-responsive genes and to examine the responsiveness of human breast cancer cells to hormone treatment. Our goal is to gain a better understanding how estrogen-responsive genes are regulated using *in vivo* ligation mediated polymerase chain reaction (LMPCR) footprinting procedures developed by Mueller and Wold (14) to examine the endogenous pS2 gene interacting with cellular proteins in its natural DNA context.

BODY

Experimental Methods

Cell Culture and RNA preparation

MCF-7 (K1) cells (15) were maintained in Minimal Essential Medium containing 5% heat inactivated calf serum. Cells were switched to serum-free Improved Minimal Essential Medium supplemented with bovine serum albumin, insulin, and transferrin (16) six days before experiments were initiated. The cells were then exposed to ethanol vehicle, 10 nM 17β-estradiol, 100 nM 4-hydroxy tamoxifen, or 100 nM ICI 182,780 for varying periods of time prior to harvest. Media was removed, 2.5 ml Ultraspec RNA lysis buffer (Biotecx Laboratories, Inc, Houston, TX) was added to near-confluent 75 cm^2 flasks and total RNA was isolated as per the manufacturer's instructions.

Northern blot analysis

RNA levels were quantitated and 15-20 μg of each sample was fractionated on a 1.5% agarose

gel. RNA was transferred to a nylon membrane and immobilized by UV crosslinking. The mRNA-bound nylon membrane was probed with ^{32}P-labeled pS2 and 36B4 DNA fragments. The pS2 and 36B4 cDNAs, generously provided by Pierre Chambon (CNRS/INSERM, Strasbourg, France), were previously described (2, 8). 36B4 encodes the human acidic ribosomal phosphoprotein PO and was used to normalize samples for unequal loading. The DNA fragments were labeled with $\alpha[^{32}P]$ dCTP using hexamer priming (17, 18). The membrane was prehybridized at 65°C for 1 hour in 5 ml of Rapid-hyb buffer (Amersham, Arlington Heights, IL.). The pS2 and 36B4 probes were boiled for 5 minutes and 500,000-750,000 cpm/ml were incubated with the prehybridized membrane for 12-16 hours at 65°C. The membrane was removed and washed twice in 2 x SSC containing 0.5% SDS for 3 minutes at room temperature and once in 0.2 x SSC with 0.2% SDS for 3 minutes at room temperature. The bands were visualized by autoradiography and quantitated using a phosphorimager and Imagequant software (Molecular Dynamics, Sunnyvale CA.).

In vivo footprinting *In vivo* DMS footprinting was carried out essentially as described by Mueller and Wold (14, 19). Cells were treated with hormone or antihormone for 2 hours and then with 0.1% DMS for 2 minutes at room temperature. For DNase I footprinting experiments, cells were treated with E_2 for 24 hours, permeabilized with NP-40 (20), and exposed to increasing concentrations (250-750 U/ml) of DNase I. Genomic DNA was isolated as described (19), except that DMS-treated DNA was lyophilized up to 8 times to remove residual piperidine. Genomic DNA (1-2 μg) was subjected to LMPCR using the linker primer oligos LMPCR1 and LMPCR2 (19) and nested primers, which annealed to DNA sequences in pS2 gene regions of interest. The primers used to examine the pS2 ERE on the noncoding strand were

Primer 1 - 5'GGGATTACAGCGTGAGCCACTGC3',

Primer 2 - 5'AAAGAATTAGCTTAGGCCTAGACGGAATGG3' , and

Primer 3 - 5'CTTAGGCCTAGACGGAATGGGCTTCAT3. The annealing temperatures used for the primers were 60°, 62°, 64°, respectively. The nested primers used to examine the TATA and CAAT regions on the coding strand were:

Primer 4 - 5'GGGCGCAGATCACCTTGCTT3',

Primer 5 - 5'GCCATTGCCTCCTCTCTGCTCC3', and

Primer 6 - 5'GCCATTGCCTCCTCTCTGCTCCAAAGG3'. The annealing temperatures for these oligos were 56°, 61°, and 67°, respectively. Footprinting experiments were repeated 3-4 times with each extract to ensure that the banding patterns observed were reproducible and at least two different extracts were used to examine the ERE and proximal promoter regions.

Results

To correlate events occurring at the level of the gene with RNA production, we first examined the level of pS2 mRNA after exposure of MCF-7 cells to varying periods of treatment with 10 nM E_2. Northern blot analysis was utilized to detect the relative levels of pS2 mRNA and constitutively-expressed 36B4 mRNA. As seen in Fig. 2, the level of MCF-7 pS2 mRNA increased after exposure to E_2 and was maximal after 24 hours of estrogen treatment. Quantitation from 4 independent determinations demonstrated that pS2 mRNA transcripts increased 16-fold after 24 hours of E_2 exposure. These findings were in good agreement with previous determinations (7, 8, 21, 22) and ensured us that the pS2 gene was essentially quiescent in the absence of exogenously added estrogen, but that it responded robustly to estrogen

treatment.

One issue of great interest was whether estrogen was required for occupation of the ERE. The pS2 ERE is located from -392 to -404 relative to the transcription initiation site (Fig. 1). This ERE (GGTCAnnnTGGCC, Ref. 21) differs from the consensus ERE (GGTCAnnnTGACC, Ref. 23) by a single nucleotide in the 3' half site. A number of *in vitro* studies have demonstrated that hormone is not required for the ER-ERE interaction to occur. Our own laboratory and others have demonstrated that the ER interacts with the ERE in the absence of hormone in gel retardation assays (24-29), promoter interference (30), and *in vitro* transcription assays (31). However, it is clear that estrogen is required for activation of estrogen-responsive genes. In order to more clearly define how the ERE is involved in regulating target genes, we examined the pS2 ERE residing in MCF-7 cells using *in vivo* footprinting. MCF-7 cells that had or had not been treated with 10 nM E_2 were permeabilized and exposed to DNase I. Genomic DNA was isolated, subjected to LMPCR procedures (19), and the ^{32}P-labeled DNA fragments were fractionated on a sequencing gel. The consensus ERE half site (GGTCA) appeared to be weakly protected in the absence of hormone (Fig. 3) though the protection at this region varied somewhat with individual experiments. However, both the consensus and imperfect (TGGCC) ERE half sites were protected when cells had been exposed to E_2.

To obtain more detailed information about the interaction of proteins with the ERE and to examine the effects of estrogen on protein-DNA interactions at the molecular level, MCF-7 cells were treated with ethanol control or E_2 and then exposed to dimethylsulfate (DMS) to methylate guanine residues that were not intimately associated with proteins. DNA was isolated, methylated guanines were cleaved with piperidine, and LMPCR procedures were carried out.

Distinct differences were apparent in the footprinting patterns when MCF-7 cells had or had not been exposed to E_2. When MCF-7 cells were exposed to ethanol control, the protection pattern in the region flanking the consensus ERE half site resembled the protection pattern of *in vitro*-methylated, naked genomic DNA except that some of the guanine residues upstream of the consensus ERE half site were protected (Fig. 4, Compare G and -- lanes). When cells were exposed to estrogen, both ERE half sites were occupied. Moreover, the pattern of protection extended ten nucleotides upstream and 21 nucleotides downstream of the ERE. In addition to the regions immediately flanking the ERE, there were also a number of differences in the protection of other more distal downstream sequences indicating that E_2 affected not only the ERE, but also affected other protein-DNA interactions in the pS2 promoter.

Another important aspect of these investigations was to examine the effects of antiestrogens on the regulation of the pS2 gene in MCF-7 breast cancer cells. Although both tamoxifen, an antiestrogen with agonistic and antagonistic properties, and ICI 182,780, a pure antiestrogen with no agonistic activity, have been used in treatment of breast cancer patients (5, 32-34), it is unclear at this point how these compounds exert their effects at the molecular level. Therefore, *in vivo* footprinting was used to examine the effects of antiestrogen treatment on protein-DNA interactions. MCF-7 cells were treated with 4-hydroxytamoxifen or ICI 182,780 and LMPCR footprinting procedures were carried out after DMS treatment and purification of genomic DNA. When MCF-7 cells were treated with either of these two antiestrogens, the guanine residue in the consensus ERE half site was protected and four of the five guanine residues flanking the consensus ERE half site were protected suggesting that proteins were interacting with most of the guanines in this region. The imperfect ERE half site was

differentially protected by these two antiestrogens. ICI 182,780 treatment also initiated changes in more distal downstream regions, one of which was altered after E_2 treatment.

Not all human breast cancer cells express ER. One would anticipate that ER-negative cells would be incapable of responding to estrogen treatment. To determine whether protein-DNA interactions were altered in the region surrounding the pS2 ERE after E_2 treatment, the ER-negative MDA MB231 cell line was examined using LMPCR footprinting. No substantial differences were detected in DNase I footprints when MDA MB231 cells had or had not been exposed to E_2 (Fig. 5). However, interesting differences were detected when MCF-7 and MDA MB231 footprints were compared. Particularly striking were hypersensitive sites in regions downstream of the ERE in MCF-7 cells, but not MDA MB231 cells. This difference in DNase I hypersensitivity is most likely due to differences in chromatin structure in these two cells lines.

Two other regions of great interest in our investigations were the TATA and CAAT regions located in the 5' flanking region of the pS2 gene (35). TATA and CAAT boxes are often present in the proximal promoters of inducible genes and are involved in formation of the basal transcription complex. Protein-DNA interactions in the TATA and CAAT regions were quite similar when MCF-7 cells had been treated with either ethanol or E_2 and then exposed to either DNase I or DMS (Figs. 6 and 7). However, hypersensitive sites were observed flanking the CAAT and TATA sequences in DNase I- treated MCF-7 cells that had been exposed to ethanol or E_2 (Fig. 6, *). The presence of hypersensitive sites in this region suggests that protein-induced conformational changes brought about by binding of transcription factors to this area may enhance the susceptibility of specific nucleotides to DNase I cleavage (36). The ability of the TATA binding protein to enhance DNA bending has been demonstrated (37) and is consistent

with this hypothesis. Interestingly, striking differences were also observed when the proximal promoter regions of the pS2 gene were examined in MDA MB 231 and MCF-7 cells (Data not shown). Varying the time of E_2 treatment had little effect on the DMS footprint (Fig. 8).

Discussion

Steroid hormone receptors exert their effects on cellular function by acting through intracellular receptors. Although a number of studies have examined the abilities of these receptors to interact with their cognate response elements residing in nucleosomally phased genomic DNA, the ability to detect these hormone-induced receptor-DNA interactions has met with varied success. When the glucocorticoid/progesterone-responsive mouse mammary tumor virus (MMTV) promoter was stably integrated into T47D human breast cancer cells, the glucocorticoid/progesterone response element was occupied after progesterone treatment, but not after glucocorticoid treatment (38, 39). When Rigaud et al. (40) examined the glucocorticoid-responsive tyrosine amino transferase (TAT) gene and failed to see protection of the glucocorticoid response element after hormone administration, they proposed that the glucocorticoid receptor functioned through a "hit and run" mechanism in which the receptor bound only transiently to the glucocorticoid response element. The transient association of the glucocorticoid receptor with the glucocorticoid response element was sufficient to disrupt nucleosomes in the TAT promoter and facilitate binding of other transcription factors to neighboring regions. In contrast, another examination of the TAT promoter demonstrated that the glucocorticoid response element was occupied after hormone treatment (41). Examination of thyroid, retinoic acid, and androgen response elements also failed to detect receptor-DNA

interactions in endogenous genes (20, 42, 43). Using *in vivo* footprinting to examine an estrogen-responsive gene, we have demonstrated using both DMS and DNase I *in vivo* footprinting that the consensus ERE half site may be weakly protected in the absence of hormone, but that both the consensus and the imperfect ERE half sites are strongly protected in the presence of estrogen. Taken together, these finding may indicate that some steroid hormone receptors interact more weakly or transiently with their response elements and that detecting this interaction may be difficult. Our findings provide evidence that the ER interacts strongly with the ERE after hormone treatment and that the receptor remains associated with the ERE for at least 24 hours after estrogen treatment.

In addition to the changes in protein-DNA interactions that occur at the ERE, multiple sites downstream of the ERE are affected by hormone and antihormone treatment. The exact location and sequence of the cis elements involved in these interactions remains to be determined. However, one could hypothesize that the factors bound to these elements may interact directly or indirectly through other adapter proteins to form the transcription complex needed for transcription to occur.

We were surprised to discover that the TATA and CAAT sequences were flanked by hypersensitive sites in MCF-7 cells before and after hormone treatment and that hormone treatment did not substantially alter the DNase I or DMS footprints observed. Thus, the basal promoter in ER-positive MCF-7 cells appears to be poised for transcription even in the absence of hormone. In contrast, in ER-negative MDA MB 231 cells, the TATA and CAAT sequences were not bounded by hypersensitive sites, nor were they affected by hormone treatments. This leads us to the intriguing possibility that the ER plays a role in organizing the basal promoter, but

that hormone is required to facilitate the multiple protein-DNA interactions required for transcription to occur.

One subject of great clinical interest has been to determine how antiestrogens, which have been successfully used to inhibit disease recurrence in breast cancer patients (5, 32-34), bring about their effects on target genes. We have examined the abilities of two antiestrogens, 4-hydroxytamoxifen and ICI 182,780, to modulate protein-DNA interactions in the pS2 5' flanking region. We observed distinct footprinting patterns when MCF-7 cells were treated with ethanol, E2, 4-hydroxytamoxifen, or ICI 182,780. Thus, 4-hydroxytamoxifen functions as partial agonist and ICI 182,780 functions as a pure antagonist because they promote association of proteins with unique regulatory elements. One would anticipate that the proteins bound to these regulatory elements would be involved in formation of unique protein-protein contacts and that the entire complex together would be responsible for mediating the effects of each compound. While this information provides us with a rather complex regulatory mechanism involving multiple cis elements and multiple, as yet unidentified proteins, it also may enable us to identify novel proteins and DNA sequences involved in mediating the effects of these antiestrogenic compounds.

Statement of Work

We have addressed several of the Technical Objectives listed in the original grant. These objectives are listed below:

Task 1-2: DNase I treatment and LMPCR conditions that result in clear footprinting ladders have been optimized to examine the pS2 gene.

Task 3: K1 cells were treated with ethanol or 17β-estradiol, DNase I treated and genomic DNA isolated. *In vivo* footprints were detected on the noncoding strand in the regions of the TATA and CAAT boxes. We will also design a set of nested primers to examine the coding strand in this region. In addition to these objectives stated in our original grant, we extended our investigations to examine dimethlysulfate *in vivo* footprints on the noncoding strand in the regions of the TATA and CAAT boxes.

Task 4: K1 cells were treated with trans-hydroxytamoxifen or ICI 182,780 and DNase I treated, but the regions of the TATA and CAAT boxes have not yet been examined.

Task 5-7: K1 cells were treated with ethanol, 17β-estradiol, trans-hydroxytamoxifen or ICI 182,780 and DNase I treated. Conditions have been optimized and *in vivo* footprints were detected with one set of nested primers designed to examine the noncoding strand of the pS2 ERE. We are in the process of optimizing conditions to examine the coding strand of the pS2 ERE using a second set of nested primers. In addition to these objectives stated in our original grant, we extended our investigations to examine dimethlysulfate *in vivo* footprints on the noncoding strand in the region of the pS2 ERE.

In addition to the objectives stated in our original grant, we extended our investigations to compared pS2 mRNA expression to events occurring at the level of the gene. We utilized both DNase I and dimethylsulfate *in vivo* footprinting, rather than just the DNase I procedure as originally proposed in the grant. Additionally, we utilized ER-negative MDA MB 231 cells so that the organization of the pS2 promoter could be compared to the organization of the pS2 promoter in ER-positive K1 cells. We felt that these were important additions that provided us with a more detailed and realistic picture of how this gene is regulated.

FIGURE LEGENDS

Fig. 1. Sequence of the human pS2 gene. The sequence of the pS2 gene from -523 to +16 (35) is shown.

Fig. 2. Effects of E2 on pS2 mRNA production in MCF-7 breast cancer cells assessed by Northern blot analysis. Cells were incubated with ethanol control or 10 nM E_2 for 0.25 to 24 hours before RNA isolation. Total RNA (10 μg) was fractionated on an agarose gel and transferred to a nylon membrane. The RNA-bound membrane was probed with ^{32}P-labeled pS2 and 36B4 DNA fragments. Equivalency of mRNA loading was monitored by normalization to levels of constituitively-expressed 36B4.

Fig. 3. *In vivo* footprinting of the pS2 ERE after exposure of intact MCF-7 cells to DNase I. MCF-7 cells were maintained in serum-free medium for six days and then treated with either ethanol control (-) or 10 nM E_2 (E_2). Cells were permeabilized with NP-40 and treated with DNase I. Genomic DNA was isolated and LMPCR was performed. The location of the imperfect and consensus ERE half sites are indicated. Naked genomic DNA samples, which had been treated *in vitro* with either DNase I (V_t) or DMS (G), were included as references.

Fig. 4. *In vivo* footprinting of the pS2 ERE after exposure of intact MCF-7 cells to DMS. MCF-7 cells were maintained in serum-free medium for six days and then treated with either ethanol control (-), 10 nM E_2,(E_2), 100 nM 4-hydroxytamoxifen (T), or 100 nM ICI 182,780 (I). Cells were exposed to DMS, genomic DNA was isolated and cleaved, and LMPCR was performed.

The location of the imperfect and consensus ERE half sites are indicated. Naked genomic DNA that was treated *in vitro* with DMS (G) was included for reference.

Fig. 5. *In vivo* footprinting of ER positive MCF-7 and ER negative MDA MB 231 human breast cancer cell lines. Cells were treated with ethanol (-) or E_2 (E_2), permeabilized with NP-40, and exposed to DNase I. Genomic DNA was isolated and LMPCR was performed. The location of the pS2 ERE is indicated. *In vitro* DNase I (V_t) or DMS (G) cleaved DNAs were included as references.

Fig. 6. *In vivo* footprinting of the pS2 TATA and CAAT sequences after exposure of MCF-7 cells to DNase I. MCF-7 cells were grown in serum-free medium for six days and then treated with either ethanol control (-) or 10 nM E_2 (E_2). Permeabilized cells were treated with DNase I. Genomic DNA was isolated and LMPCR was performed. The positions of the TATA and CAAT sequences are indicated. *In vitro* DNase I (V_t) or DMS (G) cleaved DNAs were included as references. * indicates a hypersensitive site.

Fig. 7. *In vivo* footprinting of the pS2 TATA and CAAT regions after exposure of MCF-7 cells to DMS. MCF-7 cells were grown in serum free medium for six days, treated with ethanol control (-) or E2 (E_2) and exposed to DMS. Cells were lysed, genomic DNA was isolated and cleaved, and LMPCR was carried out. The location of the TATA and CAAT sequences are indicated. *In vitro* DMS-cleaved DNA (G) was included as a reference.

Fig. 8. DMS *in vivo* footprinting of the pS2 TATA and CAAT regions after varying periods of

exposure of MCF-7 cells to E_2. MCF-7 cells were grown in serum free medium for six days, treated with ethanol control or E2 for 10, 60 or 120 minutes and exposed to DMS. Cells were lysed, genomic DNA was isolated and cleaved, and LMPCR was carried out. The location of the TATA and CAAT sequences are indicated.

-703 aagtgattct cctgacttaa cctccagagt agctaggatt acaggcaccc gcaccatgcc

-643 tggctaattt ttgtattttt ttttttgta gagacggggt ttcggccatg ttggccaggc

-583 tagtctcaaa ctcctgactt taagtgatcc gcctgctttg gcctccaaag tgttgggatt

-523 acagcgtgag ccactgcgcc aggcctacaa tttcattatt aaaaccaatt ccactgtaaa

-463 agaattagct taggcctaga cggaatgggc ttcatgagct ccttcccttc cccctgcaag

pS2 ERE

-403 **gtcacggtgg cc**accccgtg agccactgtt gtcaggccaa gccttttcc ggccatctct

-343 cactatgaat cacttctgca gtgagtacag tatttaccct ggcgggaggg cctctcagat

-283 atgagtagga cctggattaa ggtcaggttg gaggagactc ccatgggaaa gagggacttt

-223 ctgaatctca gatccctcag ccaagatgac ctcaccacat gtcgtctctg tctatcagca

-163 aatccttcca tgtagcttga ccatgtctag gaaacacctt tgataaaaat cagtggagat

CAAT box

-103 tattgtctca gaggatcccc gggcctcctt a**ggcaaat**gt tatctaacgc tctttaagca

TATA box **+1**

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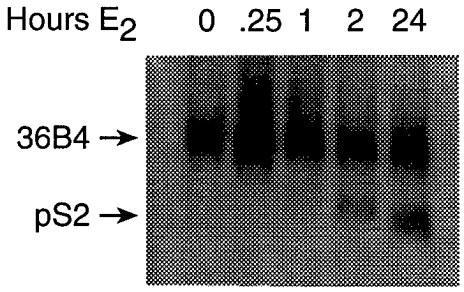
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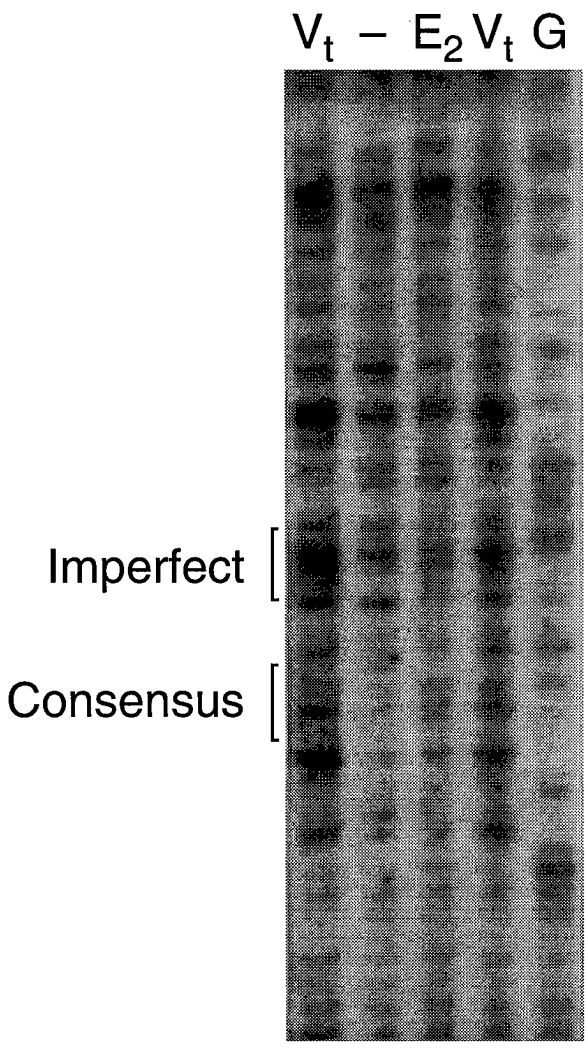
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Consensus

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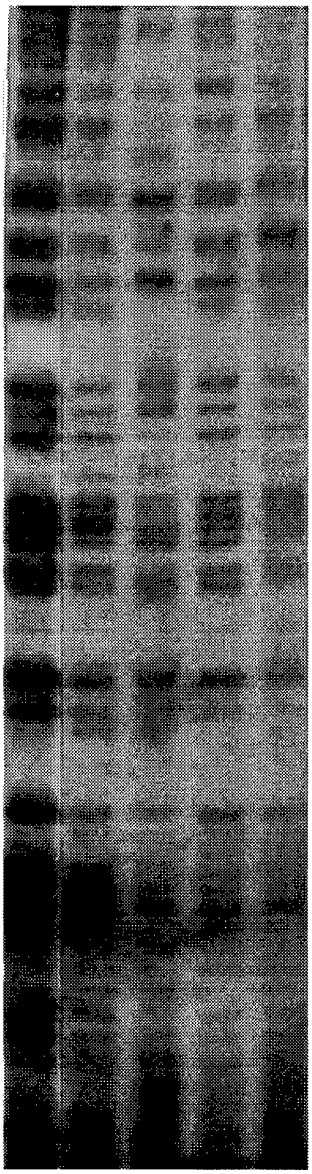

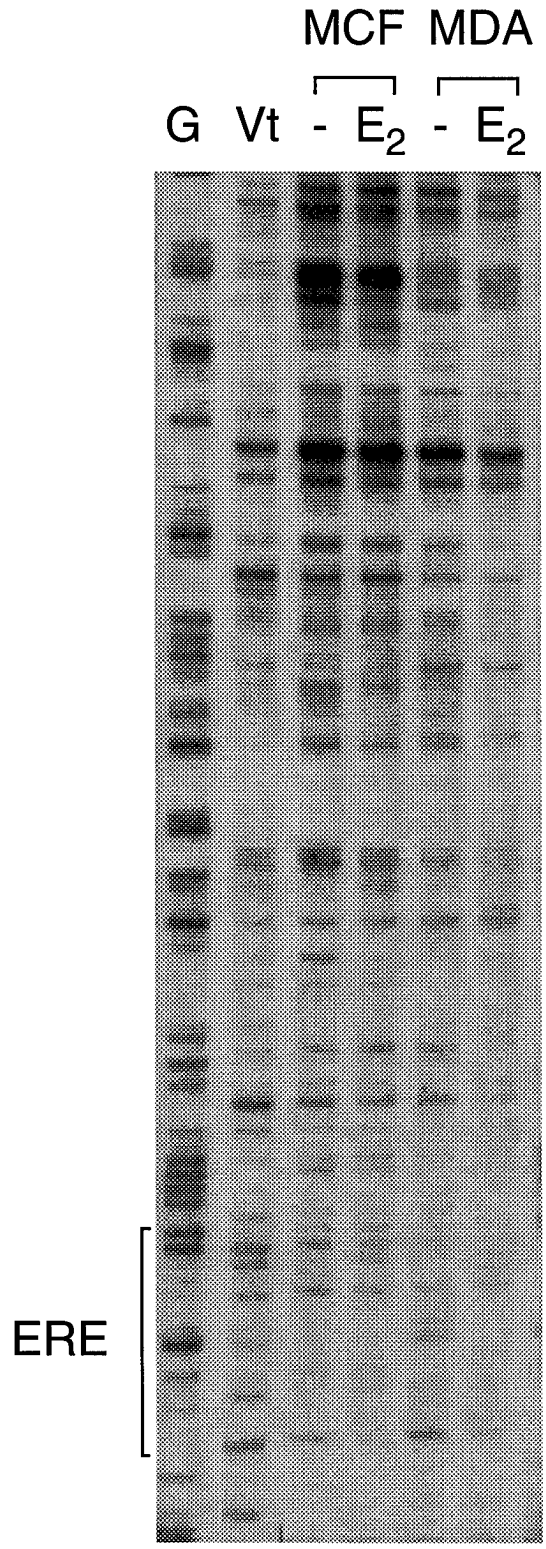
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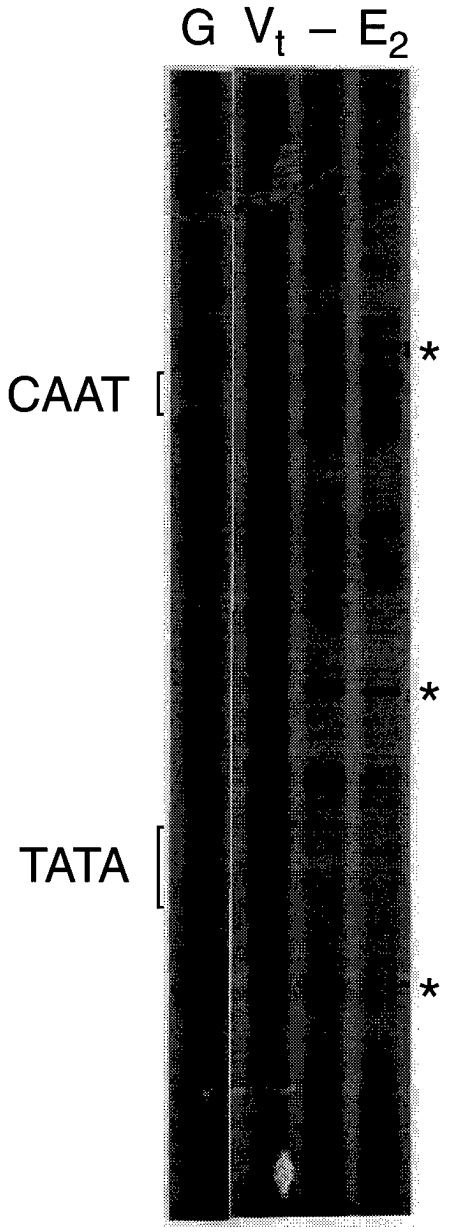
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CAAT
TATA
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*
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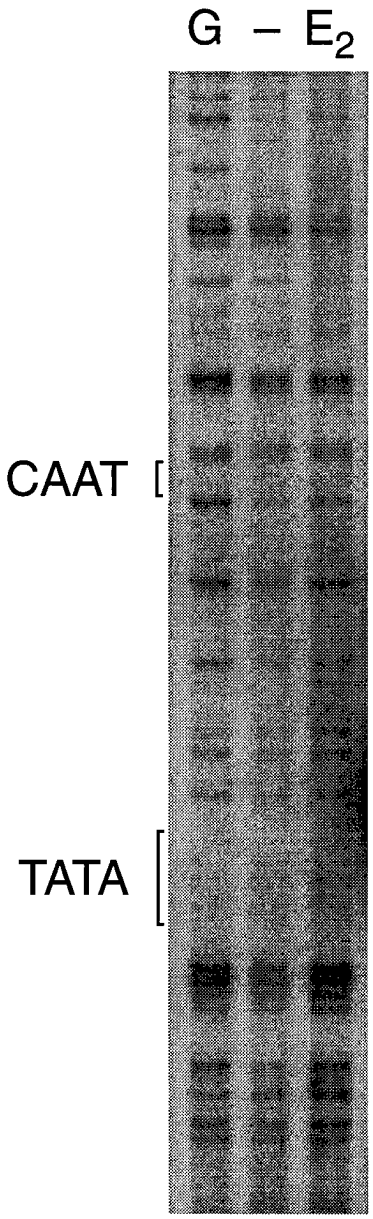
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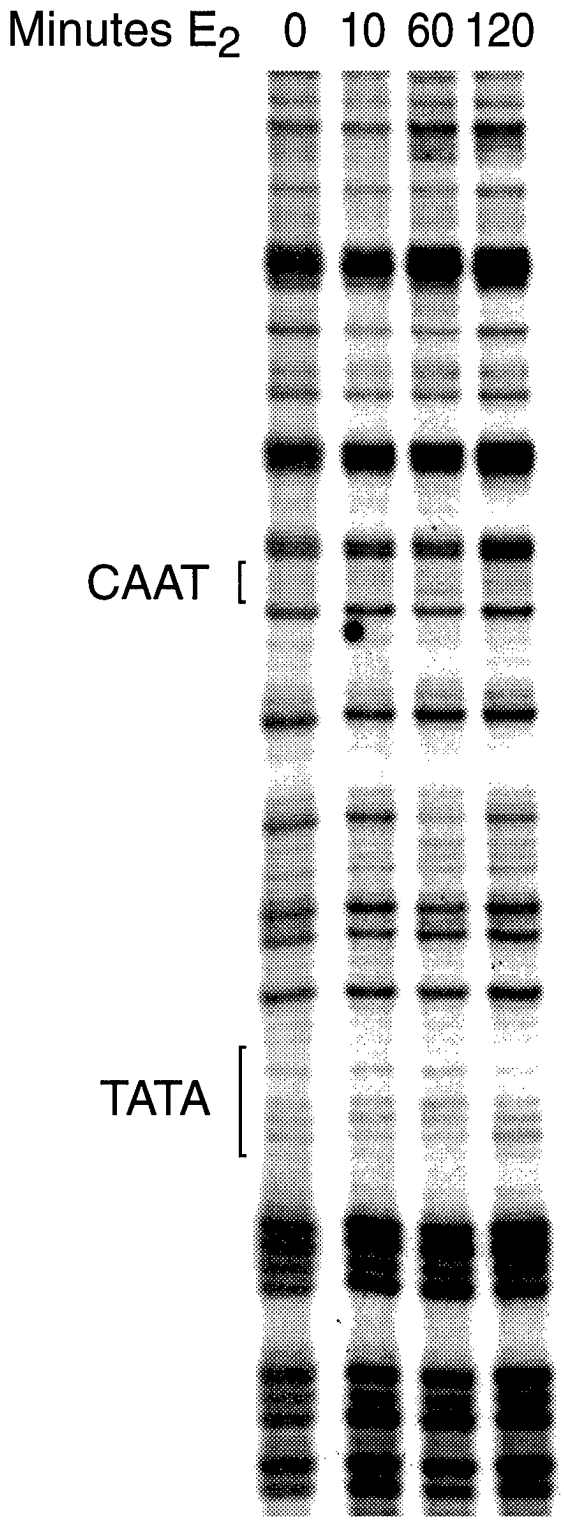
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TATA

CONCLUSIONS

1. The events occurring at the level of the pS2 gene are well coupled to the levels of pS2 mRNA in E_2-treated MCF-7 human breast cancer cells.

2. The pS2 ERE is strongly protected after estrogen treatment and remains occupied for at least 24 hours after treatment.

3. Antiestrogens mediate their effects by promoting the association of proteins with unique regions of the pS2 promoter.

4. The TATA and CAAT sequences are occupied and are flanked by hypersensitive sites in the presence and in the absence of E_2-treatment.

5. The organization of the pS2 promoter in the ER-negative MDA MB 231 cells is very different from organization of the pS2 promoter in the ER-positive MCF-7 cells.

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